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PRINCIPAL INVESTIGATOR: Wilson H. Burgess, Ph.D.

CONTRACTING ORGANIZATION: American Red Cross
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13. ABSTRACT *(Maximum 200*

The long term goal of this research program is to develop fibroblast growth factor-1 (FGF-1) through rational protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. The specific aims of this research plan remain:

1) to evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo*, and to assess their toxicological or undesirable activities
2) to generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

During the current year of support we have made significant progress with regard to these specific aims. The most important find was the demonstration that systemic FGF-1 could not only preserve but restore bone mass in animal models of osteoporosis. In addition, we were able to establish the fact that site-directed mutagenesis of FGF-1 could be used to generate mutant forms of the protein with enhanced anabolic activity for bone formation and reduced toxicological effects. This is the first demonstration of the concept that formed the basis of the original specific aims.

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William H. Burgess

PI - Signature Date

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Introduction

The long term goal of this research program remains to develop fibroblast growth factor-1 (FGF-1) via protein engineering into a potent and specific anabolic agent for the treatment of osteoporosis and fracture repair. Osteoporosis is a disease which afflicts nearly 200 million people worldwide and this number is expected to double in the next 20-30 years. It is likely that all people with the disease would benefit from treatments to increase bone mass. The greatest therapeutic challenge in the osteoporosis field at the present time is the identification of an agent that promotes significant new bone formation. Although there are effective resorption inhibitors for the treatment of osteoporosis (bisphosphanates, estrogen, and calcitonin) these drugs essentially stabilize bone mass but do not lead to substantial increases in bone mass or the restoration of trabecular bone microarchitecture. For patients with severe and established osteoporosis, there is a tremendous need for therapeutic agents that stimulate bone formation and initiate the cascade of events involved in osteoblast differentiation. Those agents that are known to have a stimulatory effect on new bone formation are fluoride, low-dose intermittent parathyroid hormone and its analogs, and the peptide growth factors that are incorporated into the bone matrix and released from bone as it resorbs.

During the past several years, it has become apparent that members of the FGF family of ligands and receptors are essential for normal skeletal growth (Li *et al.*, 1997). The preliminary data that formed the basis of the original application demonstrated a significant osteogenic potential for local and systemic administration of FGF-1 *in vivo*. The data also documented certain toxicological or undesirable effects associated with these treatments. Together these data indicated that the therapeutic window is relatively narrow. In the progress report for the first year of funding, we outlined our progress on the generation of several mutants of FGF-1 and chimeric proteins. We also reported on the *in vitro* and *in vivo* activities of these proteins. The studies confirmed that a further evaluation of existing mutants and production of additional mutants or chimeric proteins may improve the efficacy of FGF-1 as an anabolic factor in the treatment of osteoporosis.

The original specific aims of the proposal were:

1) To evaluate the effects of existing mutant forms of FGF-1 on bone cells *in vitro*, on bone formation *in vivo* and to assess their toxicological or undesirable effects.
2) To generate additional FGF-1 mutants or chimeric proteins that are likely to exhibit enhanced anabolic activity on bone with reduced toxicological effects.

Progress towards these aims achieved during the previous and current funding period are summarized in the following report.

Body

In the progress report for the first year of support we described the solubility problems we encountered with a variety of the mutant FGF-1 constructs (protein ended up in inclusion bodies of bacteria). Although a solution to the purification of these proteins was provided, we have observed variability in the biological activities of the urea solubilized proteins. Although this is not a critical problem to the long term goals of the study, it is an existing problem that impacts the progress of the work. The take home message is that even for previously characterized mutants of FGF-1, the *in vitro* biological activity of the proteins must be validated prior to systemic or other *in vivo* assays. Despite this inconvenience, significant progress has been made during the second year of support. This variability in the specific activity of the recombinant proteins is the only significant problem encountered during this year of support.

The primary focus of the current year has been on the cys-free mutant of FGF-1. During the first year of support, we established that this mutant FGF-1 was ~10 fold more potent than the wild-type protein in maintaining trabecular bone mass in ovariectomized rats. Although these findings were encouraging, they did not address directly the heart of the real problem which is restoration of bone mass. Our approach to this problem has been to delay administration of FGF-1 or FGF-1 mutants until three months post-ovariectomy, at which time, significant loss of trabecular bone has occurred. This model for restoration of bone mass has occurred. This model for restoration of bone mass has been the focus of all of the experiments conducted during this funding period. There are

serious concerns regarding long term systemic administration of any polypeptide growth factor. We believe that systemic FGF-1 treatment will be relatively brief to restore bone mass. Once the increased bone mass is established, current therapies to maintain bone mass should be effective in management of the disease.

We have also begun a detailed analysis of the effects of systemic FGF-1 on other organs or tissues. The gross analysis (appearance, wet weight) did not indicate significant pathology associated with the treatments. A more detailed examination of various organs revealed potentially adverse effects on the kidney (see below). A second advance in our analysis of the *in vivo* data relates to quantitation of the restoration of bone mass in the ovariectomized animals. We were able to purchase (with institutional funds) a Bioquant image analysis hardware and software package. One of the members of my laboratory, Lawyna Holland, is the primary operator of the system. She is currently analyzing all of our H and E stained tissue sections from previous systemic studies in order to quantitate the bone mass in the different treatment groups. It should be noted that our ability to conduct significant histomorphometric analysis of our studies was one of the few concerns raised in the initial peer review of our application. In summary to this general introduction, we believe we have maintained or surpassed the original statement of work for the first two years. We have encountered several problems and have solutions in place. We have also improved our ability to analyze existing data and have begun a detailed analysis of the toxicology of the mutants that appear to be the most effective anabolics for bone. Although it is beyond the scope of a progress report to summarize all of the data, documentation of what we consider to be the most significant findings is provided below.

Systemic Treatment of Aged Ovariectomized Rats: The major challenge in the treatment of osteoporosis is the restoration of lost bone mass. In the previous progress report, we demonstrated that FGF-1 would effectively prevent the loss of bone mass following ovariectomy. In the current year, we have allowed animals to lose bone mass by delaying systemic treatment until 4.5 months following ovariectomy. The results of some of these studies are provided on appendices 1-4. At 4.5 months, animals were given 200μg/kg

FGF-1 by tail vein injection for 35 days on a 5 days on, 2 days off dosing. Two weeks after the last injection animals were euthanized and tissue was analyzed. The figures show H and E or toludine blue staining of frontal sections through the tibias of these animals.

Appendix 1. H and E stain of normal rat, rat 4.5 months after ovariectomy (OVX) and an ovariectomized rat given FGF-1 starting at 4.5 months. The dramatic loss of trabecular bone is apparent in the OVX animal. A break can be seen in the cortical bone that occurs frequently during sectioning of tissue from these animals. In contrast, 5 weeks of systemic FGF-1 restores significant mass to the trabecular bone of these animals. This loss and restoration is more apparent in the higher magnification of Appendix 2. It can also be seen from this figure that the cartilage of the growth plate is normal in all the animals. This is also clear from the toludine blue stained figure in Appendix 3. Appendix 4 illustrates something that we observed for the first time this year and provides an explanation for the loss of strength in the cortical bone of the tibial shaft. The figure shows toludine blue stained sections of the remodeling stacks in the shaft. There are areas of bone resorption and formation that account for the total turnover of bone that occurs every 3 years or so. In the normal rat the bone adjacent to the cartilage stacks is cellular. In the OVX rats adjacent bone is acellular with an amorphous osteoid appearance. A dramatic increase in the cellularity and structure of bone adjacent to the stacks is apparent in the OVX animals receiving FGF-1. These date demonstrate that the anabolic effects of systemic FGF-1 are not limited to bone easily accessible from the marrow.

Cys-free Mutant of FGF-1: In the original application we provided *in vitro* data to suggest that the cys-free (3 serines changed to cysteine) mutant of FGF-1 was ~10 fold more potent as a mitogen for osteoblastic cells than the wild-type protein. During the current year of support we conducted a systemic study of the wild-type and mutant FGF-1 in the aged OVX animal model described above. We treated animals with 200 or 20μg/kg of wild-type or cys-free mutant. H and E stains of sections from the tibia of these animals are shown in Appendix 5. The figure shows the results obtained using

20μg/kg of the two proteins. At 20μg/kg the wild-type FGF-1 is not effective in restoring trabecular bone mass whereas significant trabecular bone can be seen in the cys-free treated animals. The results are seen more clearly at a higher magnification (Appendix 6). It should be noted that these were random fields selected by a technician who was blind to the study. Obviously we need to qualify the bone mass in these treatment groups. Such studies are in progress using the Bioquant system described previously.

Pathology/Toxicology: A weakness of the original application was the lack of detail regarding assessment of undesirable effects of systemic therapy on other organs. During the current year of support, we have addressed this weakness by conducting histological examinations of organs collected from the various treatment groups. A variety of unrelated experiments had established that FGF-2 (basic FGF) injections into normal mice induced renal glomerular capillary injury. Studies were conducted to determine whether systemic administration of FGF-1 induced similar changes in rats. Renal tissues were processed for electron microscopy studies. The ultrastructural changes were evaluated blindly by a collaborator, Patricio Ray, M.D., of the Children's National Medical Center at no cost to the program. The histological changes were scored on a semiquantitative scale of 0 (normal tissue) to 3 (most changes). Panels A-F of Appendix 7 show representative micrographs of the glomerular ultrastructural capillary changes observed.

A) Control rats: Normal renal glomerular capillaries and glomerular basement membranes (GBM, arrows). Score 0

B) Control ovariectomized rats: Normal renal glomerular capillaries and GBM. Score 0

C) Ovariectomized rats injected with 20μg/kg/day mutant aFGF. Slight focal increase in the GBM thickeners (arrows). Score 0.5

D) Ovariectomized rats injected with 20μg/kg/day aFGF. Slight focal increase in the GBM thickness, endothelial and mesangial matrix expansion. Focal and slight effacement of the glomerular foot processes (arrows). Score 1

E) Ovariectomized rats injected with 200μg/kg/day mutant aFGF. Slight wrinkling of the GBM and increased focal thickness of the GBM. Score 2

F) Ovariectomized rats injected with 200μg/kg/day aFGF. Folding, wrinkling and slight thickening of the GBM. Increased mesangial matrix expansion and increased interstitial space between renal cortical tubules. Score 3

Together the data obtained on restoration of bone mass and reduced toxicology/pathology of the cys-free mutant relative to wild-type protein represent the first proof of principle that FGF-1 can be engineered at the sequence level to generate a more specific and potent anabolic agent for bone. The concept that the protein could be mutated to improve its desire while reducing the undesired effects was the crux of the original application. Plans for the next year of funding will focus on quantitation of these results and the generation of additional mutants based on the cys-free or HBGAM-chimera of FGF-1.

Studies on Alternative Administration of FGF-1: Two studies were conducted to evaluate methods to improve the treatment regimen to one that was more compatible with standard care. The first utilized intramuscular injection of FGF-1 in the OVX rats. These treatments were not effective in restoring bone mass and resulted in morbidity at the sites of injection. The second study evaluated a one week on/one week off treatment with FGF-1. The preliminary histological analysis of this treatment indicates that the on/off treatment is as good or better than continuous therapy. Quantitation of these studies are in progress.

Conclusions

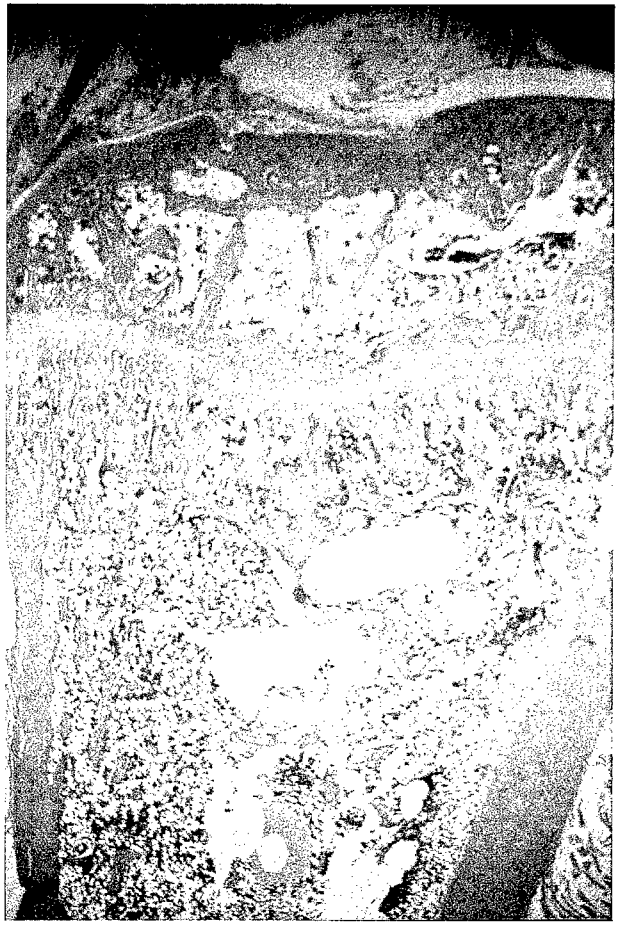
In summary, progress towards the specific aims of the original application has been steady and consistent with or ahead of the original statement of work. The data establishing the ability of systemic FGF-1 to restore bone mass in aged OVX animals is impressive relative to any published treatment. The demonstration that site-directed mutagenesis can be used to enhance a desired activity while reducing untoward side effects is unique in the FGF-1 field. The data will benefit from the quantitative analysis

we have initiated. The results of the past year provide significant enthusiasm for the concept of improved anabolic performance of FGF-1 for bone through additional mutagenesis studies.

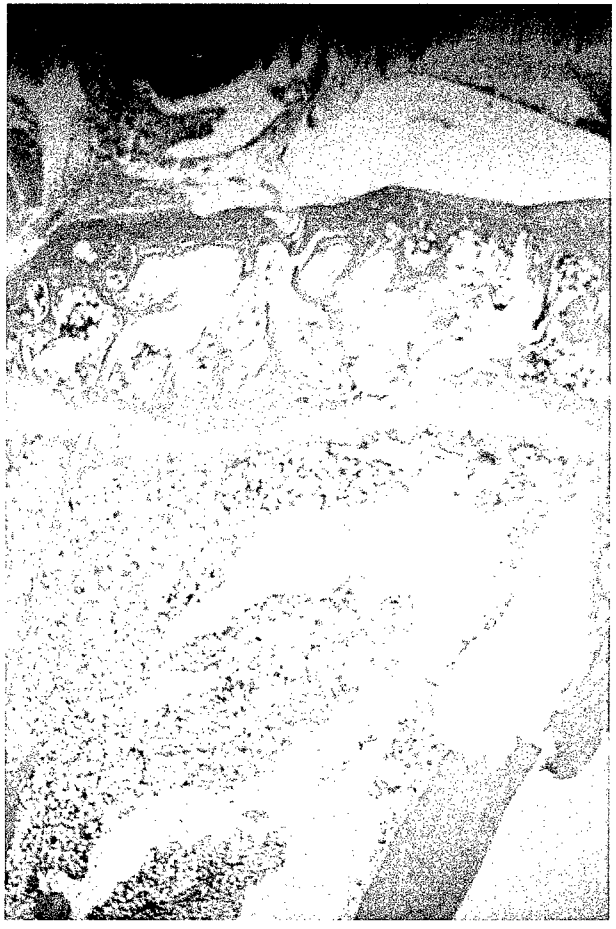
References

Y. Li, K. Mangasarian, A. Mansukhani, C. Basilico. *Oncogene*. **14**, 1397 (1997).

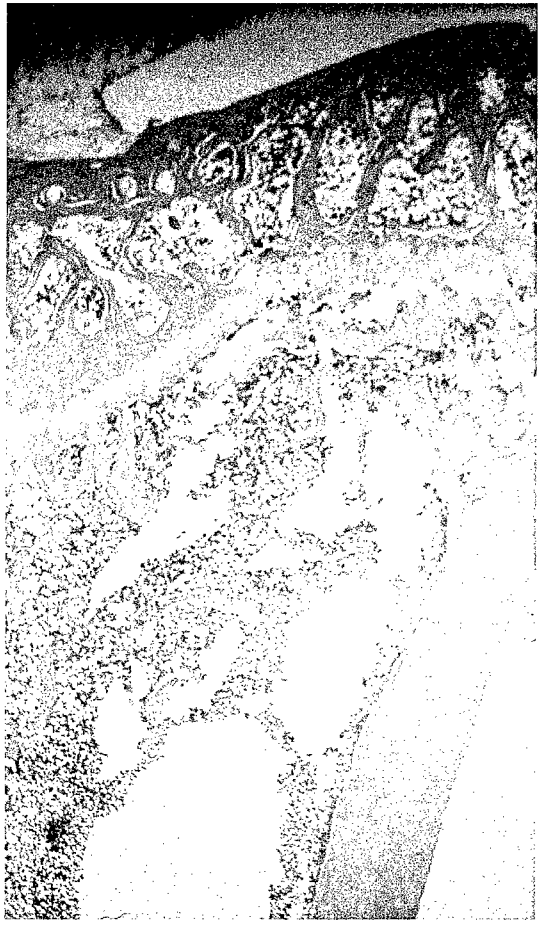
Normal Rat

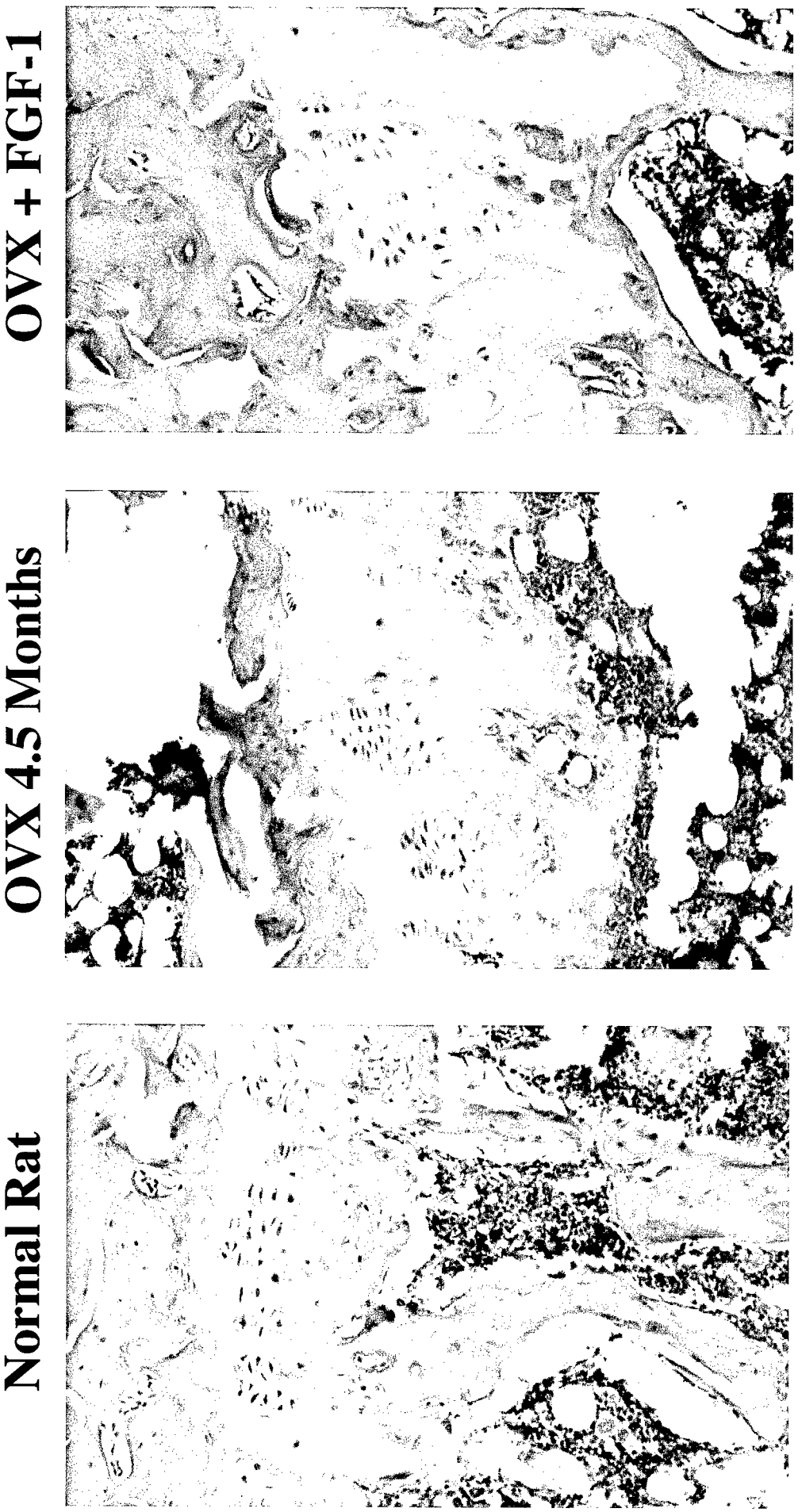


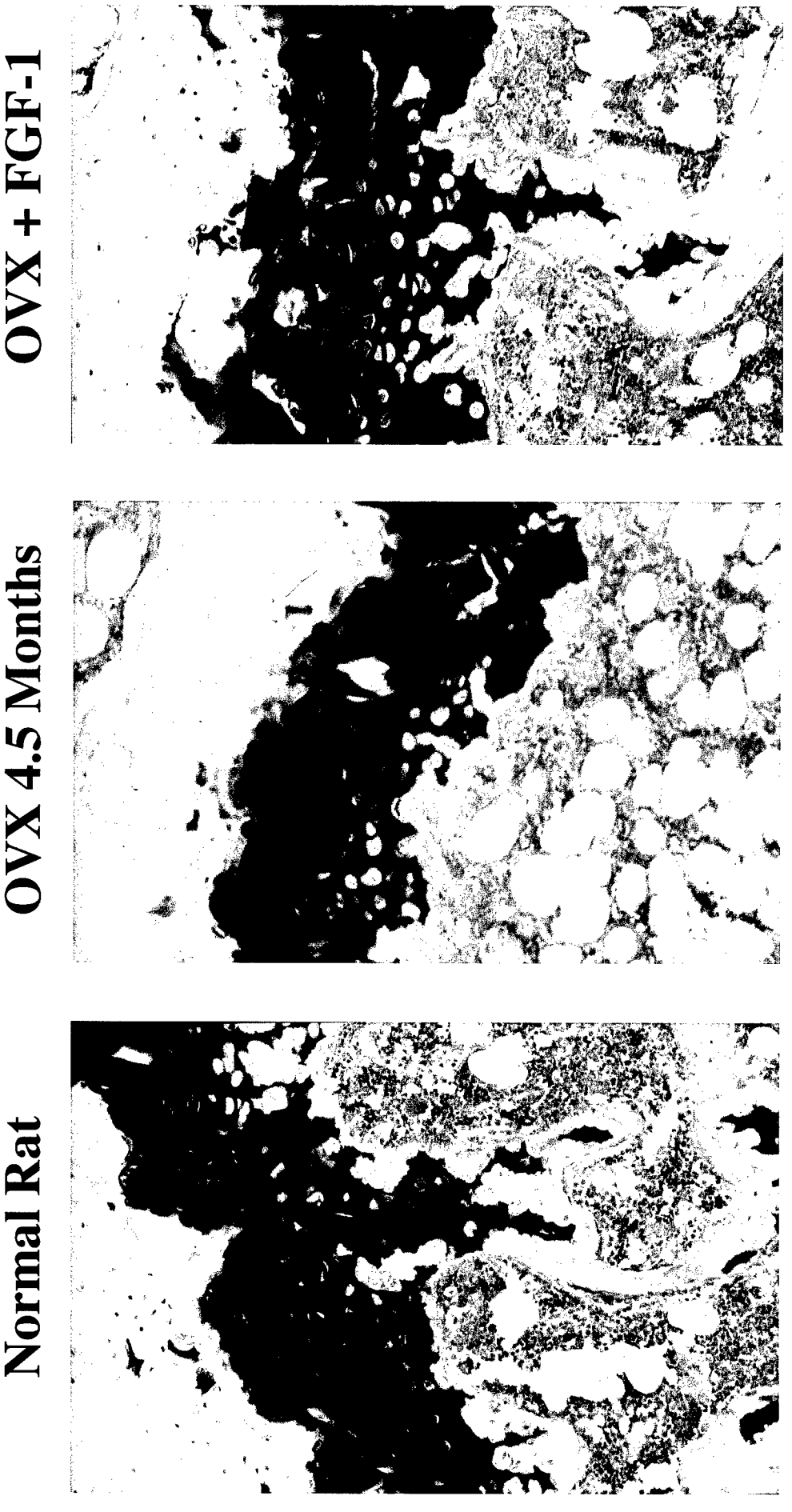
OVX 4.5 Months



OVX + FGF-1




Normal Rat
OVX 4.5 Months
OVX + FGF-1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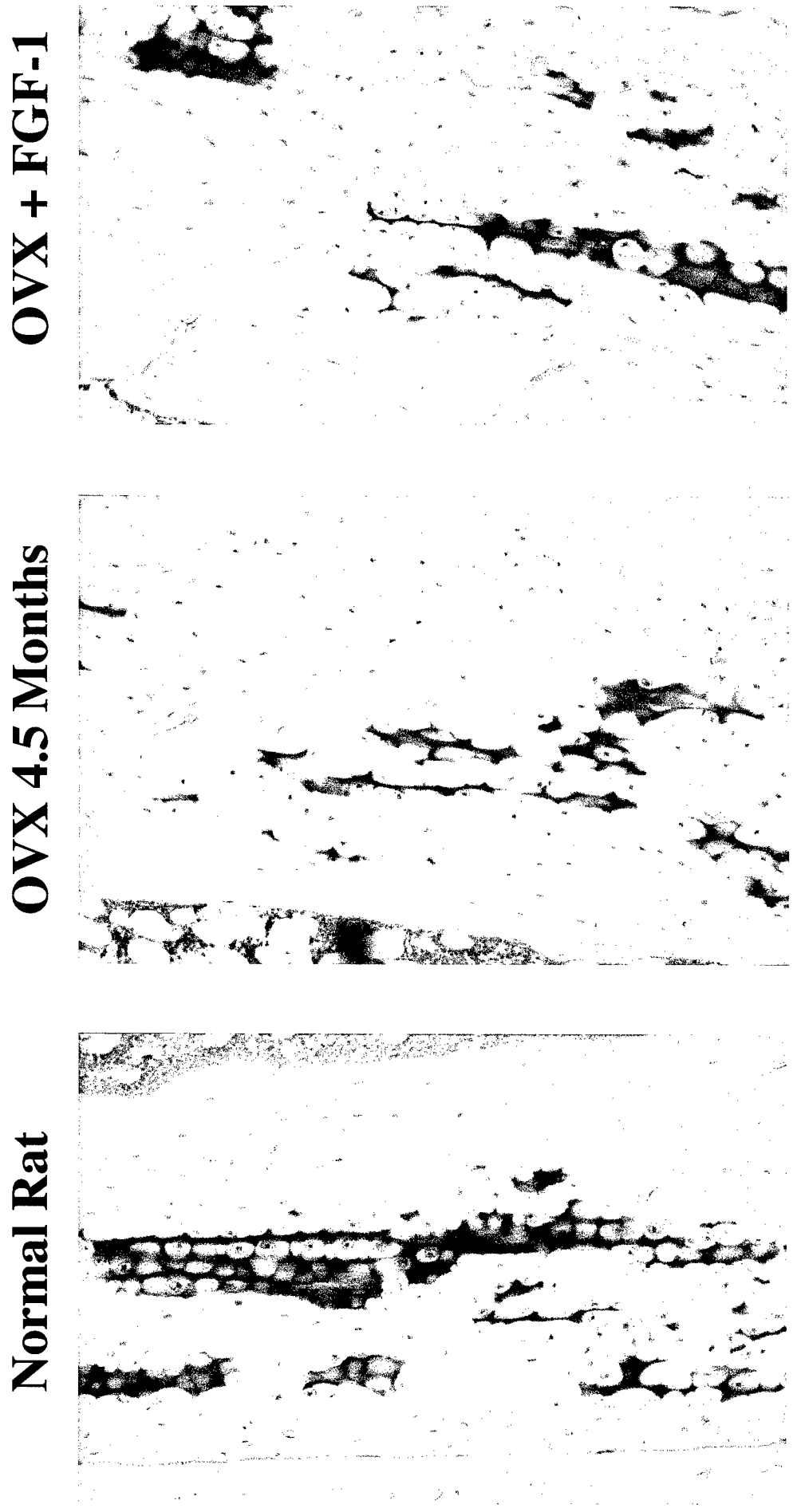
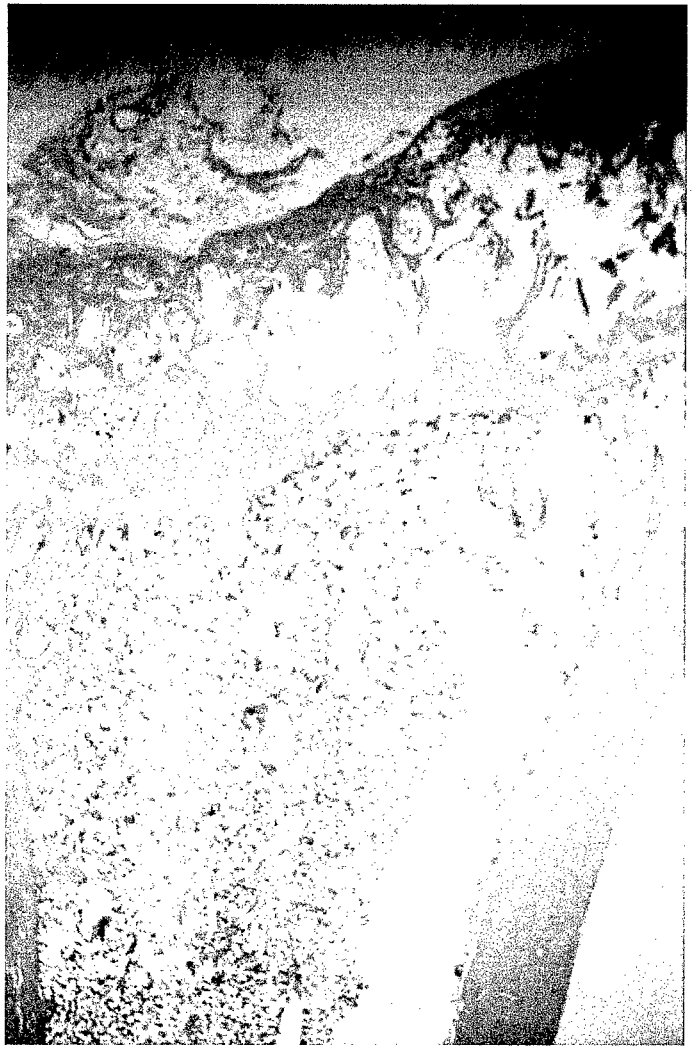
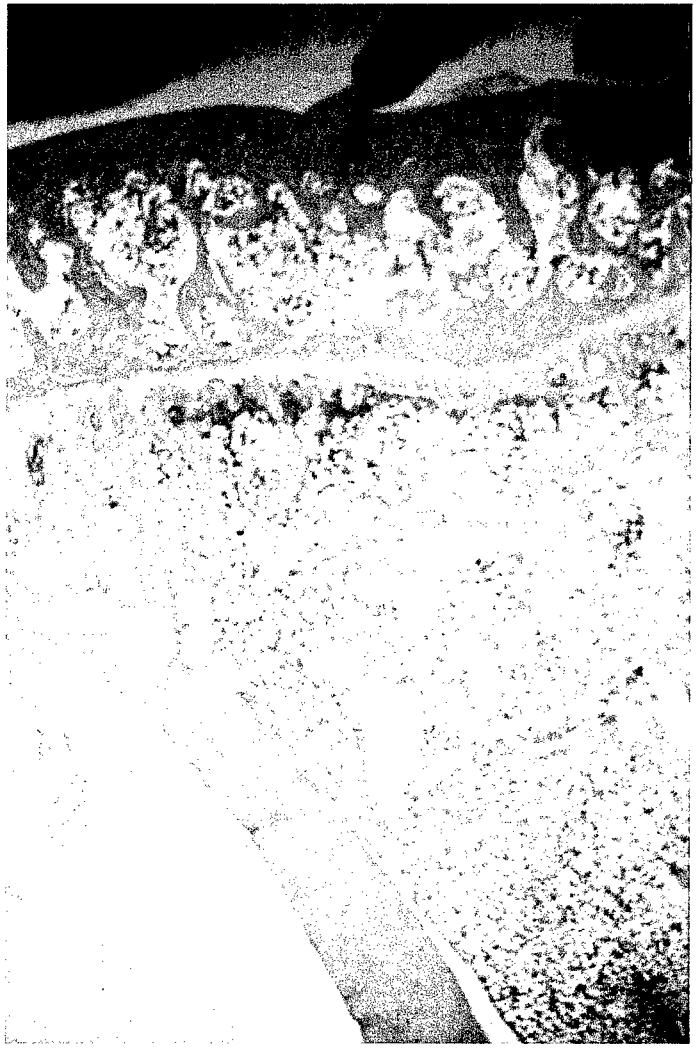
OVX 4.5 Months
OVX + FGF-1


OVX + FGF-1
OVX 4.5 Months
Normal Rat

Wild-Type FGF-1



Mutant FGF-1



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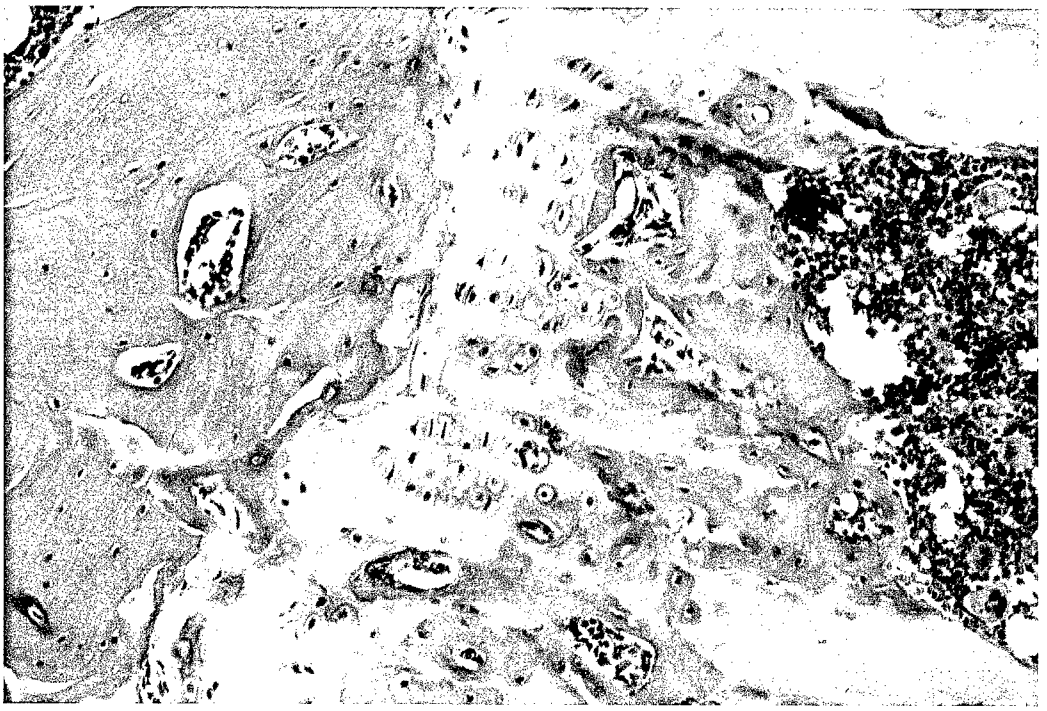

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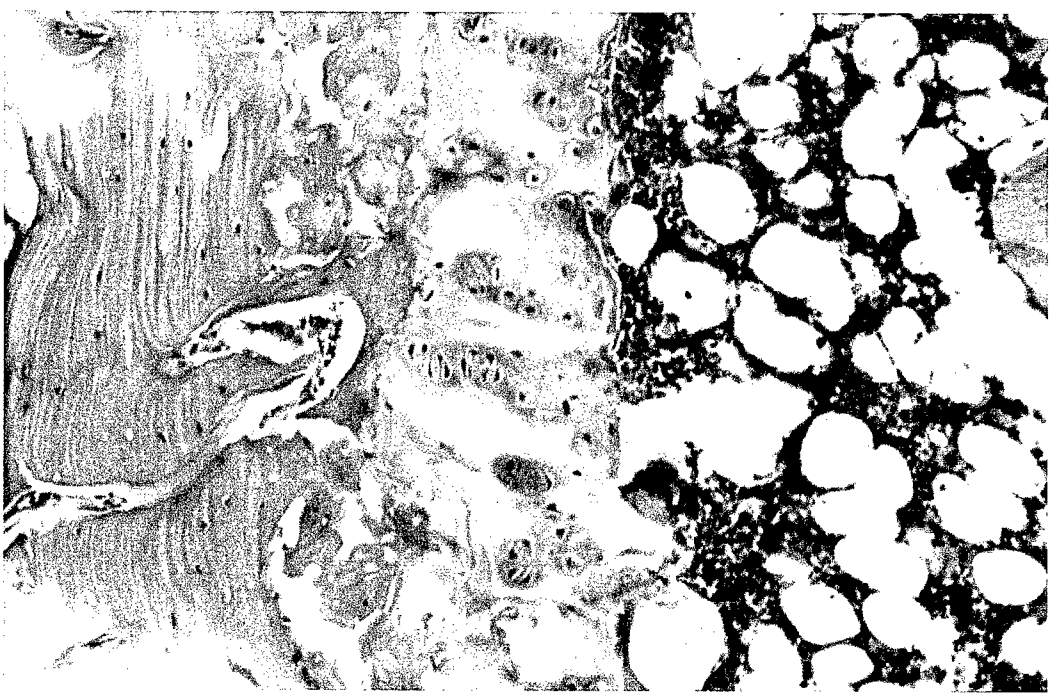

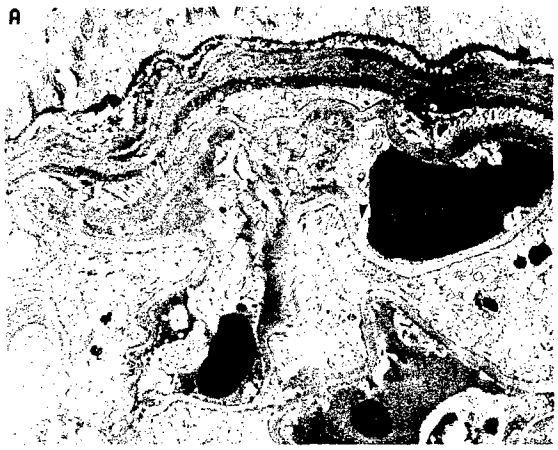
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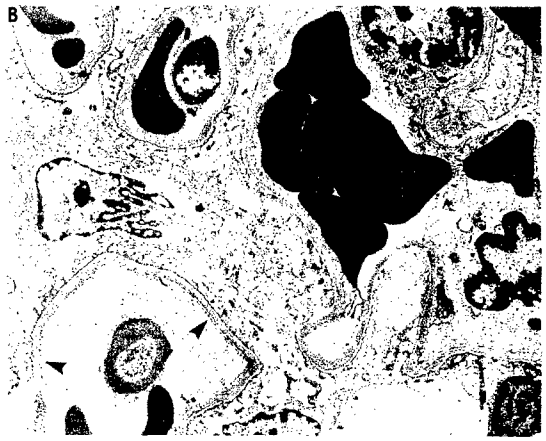
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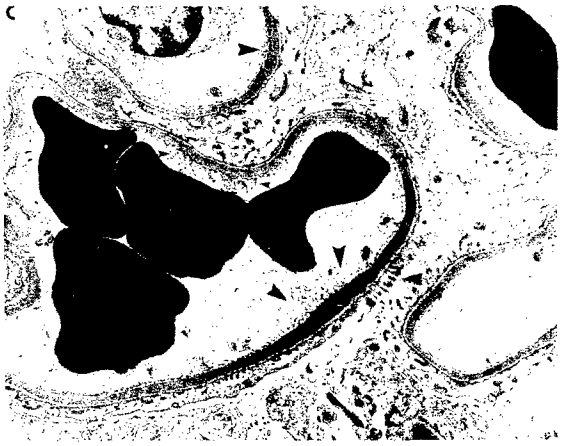
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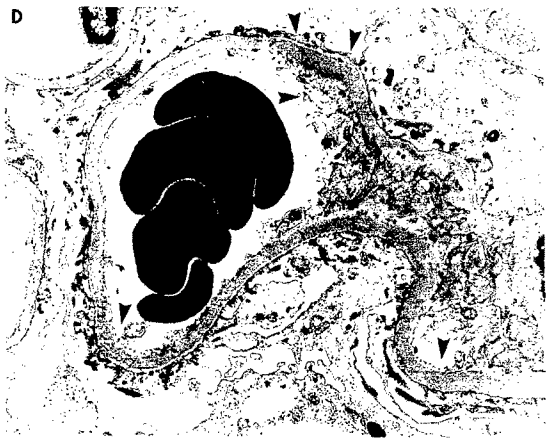
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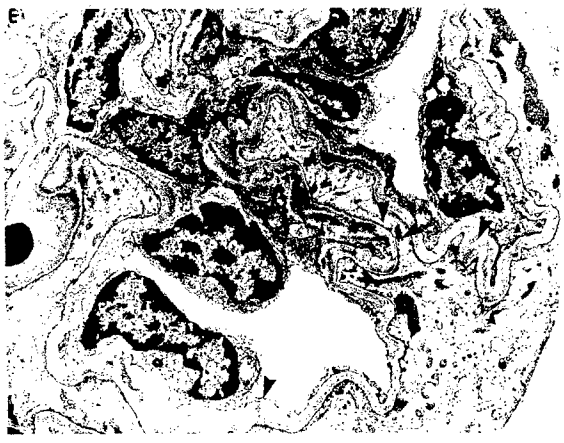
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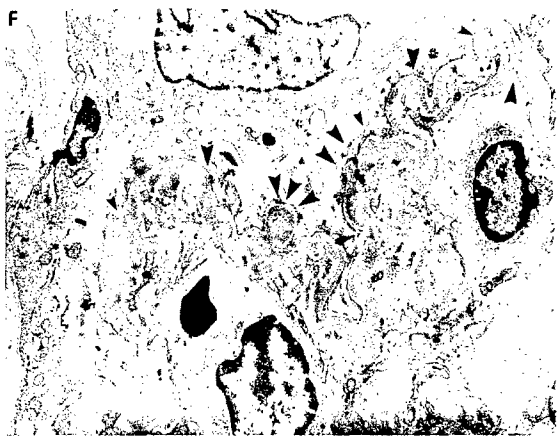
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